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Short Communication

Caveolin-1 Is Down-Regulated in Human Ovarian Carcinoma and Acts as a Candidate Tumor Suppressor Gene

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To identify novel markers differentially expressed in ovarian cancer *versus* normal ovary, we hybridized microarrays with cDNAs derived from normal human ovaries and advanced stage ovarian carcinomas. This analysis revealed down-regulation of the *caveolin-1* gene (*CAV1*) in ovarian carcinoma samples. Suppression of *CAV1* in ovarian carcinomas was confirmed using a tumor tissue array consisting of 68 cDNA pools from different matched human tumor and normal tissues. Immunohistochemistry demonstrated expression of caveolin-1 in normal and benign ovarian epithelial cells, but loss of expression in serous ovarian carcinomas. In low-grade carcinomas, redistribution of caveolin-1 from a membrane-associated pattern observed in normal epithelium to a cytoplasmic localization pattern was observed. No expression of caveolin-1 was detectable in four of six ovarian carcinoma cell lines investigated. In SKOV-3 and ES-2 carcinoma cells, which express high levels of the caveolin-1 protein, phosphorylation of the 22-kd caveolin-1 isoform was detected. Inhibition of both DNA methylation and histone deacetylation using 5-aza-2′deoxycytidine and Trichostatin A, respectively, relieves down-regulation of caveolin-1 in OAW42 and OVCAR-3 cells which is in part mediated by direct regulation at the mRNA level. Expression of *CAV1* in the ovarian carcinoma cell line OVCAR-3, resulted in suppression of tumor cell survival *in vitro*, suggesting that the *CAV1* gene is likely to act as a tumor suppressor gene in human ovarian epithelium. *(Am J Pathol 2001, 159:1635–1643)*

Tumor formation and progression is a multistage process based on numerous genetic alterations.[1] Proto-oncogenes such as *RAS* and *MYC* can be mutationally activated or amplified, tumor suppressor genes such as *p53*, *p16*, and *RB* are inactivated by mutations, by genomic silencing, or deletion.[2–4] These genetic alterations have a fundamental impact on signal transduction control, cell cycle regulation, and apoptosis. In normal cells, transmission of growth factor signals from their membrane receptors to the nucleus is a highly organized process that involves controlled spatio-temporal activation of signaling molecules. This is in part accomplished by the formation of caveolae, membrane invaginations of vesicular shape observed in many cell types.[5] Caveolae have been shown to play a role in transcytosis and lipid metabolism, but also in signal transduction. They are enriched in lipids, membrane receptors, and a large number of signaling molecules among which have been found RAS and RAF, SHC, PKCα, and mitogen-activated protein kinase. The caveolin proteins (caveolin-1α, -1β, 2, and 3) are the major integral protein components of membrane caveolae.[6] Caveolin-1 has been shown to bind to the HRAS protein, the EGF-receptor, SRC family tyrosine kinases, and protein kinase C.[7–9] These observations have led to the hypothesis, that Caveolins act as docking proteins that concentrate signaling molecules at distinct membrane domains in normal cells.[6] In addition, a critical role for caveolin-1 to act also as a signaling regulator has been suggested by the observation that targeted down-regulation of *caveolin-1* in NIH3T3 cells is sufficient to activate mitogen-activated protein kinase and stimulate anchorage-independent growth.[10] Loss of caveolae formation on transformation by oncogenes has been described earlier[11] and the caveolin-1 protein was reported

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to be phosphorylated or even lost in transformed cells. As shown recently, caveolin-1 also inhibits c-Neu-dependent signaling in mice and loss of caveolin-1 expression from human mammary carcinoma cell lines suggested a role for the protein in human breast cancer.[12] Although the potential of caveolin-1 to restrain tumor cell growth has been demonstrated,[13,14] only a few studies investigated the expression of caveolin-1 in normal and malignant human epithelial and mesenchymal cells *in situ*.[15–17]

Our microarray-based approach to identify genes differentially expressed in human ovarian carcinoma as compared to normal ovary revealed down-regulation of the *caveolin-1* gene (*CAV1*) in ovarian carcinomas. In addition, we used a second, tumor tissue-specific array to investigate *caveolin-1* expression in 68 different matched cDNA pools. By immunohistochemistry, we further confirmed expression of the caveolin-1 protein in the epithelial cells of the normal ovary but not in the ovarian carcinoma cells and investigated the mechanism of down-regulation of the *CAV1* gene.

Materials and Methods

Tumor Specimens

Human ovarian tissue specimens used for RNA preparation were snap-frozen in liquid nitrogen. Immunohistochemical analysis was conducted on formalin-fixed, paraffin-embedded specimens of normal ovaries, benign ovarian tumors, and ovarian carcinomas selected from the archives of the Institute of Pathology, University Hospital Charité, Berlin. Histopathological diagnosis of the tumors was performed according to the World Health Organization classification. The tissues included 6 normal ovaries, 19 serous cysts or serous adenomas, 7 mucinous adenomas, 5 serous ovarian tumors of borderline malignancy, 29 serous carcinomas (3 grade 1, 10 grade 2, 16 grade 3), 2 grade 1 mucinous carcinomas, and 3 grade 2 endometrioid carcinomas.

RNA Preparation

Frozen tissue sample were homogenized in liquid nitrogen and dissolved in lysis buffer. RNA was prepared using the Atlas pure RNA system according to the protocol supplied by the manufacturer (Clontech, Palo Alto, CA) and controlled for DNA contamination. RNA from the cell lines was prepared according to the method of Chomzcynski and Sacchi.[18]

Expression Analysis Using the Tumor Tissue Array

The matched tumor/normal expression array consists of 68 cDNAs, synthesized from human tumorigenic and corresponding normal tissue (*http://www.clontech.com/techinfo/manuals/pdf/pt3424–1.pdf*). Each pair was independently normalized based on the expression of three housekeeping genes and immobilized in separate dots.[19] A *CAV1*-specific cDNA fragment was radiolabeled using a DECA-primeII labeling kit (Ambion, Austin, TX), hybridized overnight at 68°C using ExpressHyb Hybridization Solution (Clontech), washed, and exposed to Biomax MS X-ray film with an intensifying screen (Eastman Kodak Co., Rochester, NY). Signal intensities were calculated for individual spots using a STORM-860 phosphoimager (Molecular Dynamics, Eugene, OR).

Immunohistochemistry

Two-μm sections cut on silane-coated slides were deparaffinized and boiled for 5 minutes in 10 mmol/L sodium citrate buffer, pH 6.0. The mouse monoclonal anti-caveolin-1 antibody (clone 2297; Transduction Laboratories) was applied for 1 hour at a dilution of 1:500. To ensure consistent staining intensities, normal mesenchymal tissue at the periphery of each tumor specimen and the capillary endothelial cells within the tumors were examined as positive controls. In negative controls, the primary antibody was omitted or replaced by an antibody with irrelevant specificity (mouse IgG1, X0931; DAKO). Immunostaining was accomplished using a Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA) as recommended by the manufacturer. The sections were counterstained with hematoxylin and mounted in Permount. Digital images were acquired using an Olympus DP-10 charge-coupled device camera. Adjustments in image contrast were performed identically on the images. The caveolin-1 immunostainings were evaluated using a semiquantitative estimation. A composite score was obtained by multiplying the values of the mean staining intensity and the percentage of caveolin-1-positive cells. The intensity was graded as 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive, equivalent to the normal mesenchymal cells of the surrounding stroma).

Cell Culture

The human ovarian carcinoma cell lines SKOV3, ES-2, OAW42, CAOV-3, MDAH 2774, and OVCAR3 were maintained in Dulbecco's modified Eagles Medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum and 2 mmol/L glutamine. Human ovarian surface epithelial cells were cultivated in a 1:1 mixture of medium 199 (Sigma Chemical Co., St. Louis, MO) and MCDB105 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum and 2 mmol/L glutamine. Trichostatin A (TSA) (Sigma) was applied at 25 ng/ml, 5-aza-2′deoxycytidine (Sigma) at 5 μmol/L, the MEK1 inhibitor PD98059 (Alexis, San Diego, CA) at 50 μmol/L, the PI-3 kinase inhibitor LY294002 (Alexis) at 10 μmol/L. Methyl sulfoxide was used as a solvent control for PD98059, Ethanol was used as a solvent control for LY294002 and TSA. Cells were incubated for 3 days before preparation of cell extracts or RNA.

Expression Constructs

The pLNHX vector (Clontech) and pLNHX-caveolin-1 expression vector was described recently.[15] In the

pc-CAV-V5 plasmid (Genestorm; Invitrogen, Groningen, Netherlands), expression of *caveolin-1* is controlled by the cytomegalovirus immediate early promoter and fused to a V5 epitope Tag.

Colony Formation Assays

In stable transfections, 2×10^5 OVCAR-3 cells per 6 well plate were transfected with 1.5 μg of pLNHX-caveolin or the empty vector using Fugene (Roche, Mannheim, Germany) according to the protocol of the manufacturer. Seventy-two hours after transfection, G418-resistant clones were selected by the addition of G418 at 0.5 mg/ml to the culture medium for 10 days. Several individual clones were further expanded and analyzed. Stable transfections using the pc-CAV-V5 plasmid or the empty vector pcV5 were performed in a similar way but selection was performed using 30 μg/ml Zeocin (Invitrogen) for 10 to 14 days.

Terminal dUTP Nick-End Labeling (TUNEL) Assays

For the detection of apoptosis, 1×10^6 OVCAR-3 cells per 10-cm^2 dish were transiently transfected with 3 μg pc-CAV-V5 or the empty vector pcV5 using Fugene (Roche). Forty-eight hours after transfection cells were fixed in 4% formaldehyde and processed for a TUNEL assay using the ApoAlert DNA fragmentation kit (Clontech) as described by the manufacturer.

Northern Blot Analysis

For Northern blot preparation, 10 μg of total RNA were separated by electrophoresis through a 1.2% agarose gel and blotted onto Hybond N nylon membrane (Amersham, Arlington Heights, IL). A *CAV1*-specific cDNA fragment was hybridized at 58°C for 2 hours using ExpressHyb Hybridization Solution (Clontech), washed, and exposed to Biomax MS X-ray film with an intensifying screen (Eastman Kodak Co) for 2 days.

Western Blot Analysis

Protein extracts were prepared by incubation of 2×10^6 cells in 500 μl of RIPA buffer (150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mmol/L Tris-HCl, pH 8.0, 100 μmol/L sodium orthovanadate, 2 mmol/L aprotinin) for 30 minutes on ice. Tissue samples were homogenized in the same buffer. Sample buffer (2×) was added to the cell extract (120 mmol/L Tris-HCl, pH 6.8, 200 mmol/L dithiothreitol, 4% sodium dodecyl sulfate, 20% glycerol, 0.002% bromphenol blue), samples were boiled for 10 minutes, centrifuged, and aliquots were stored at −20°C. Ten μg of each extract were used and processed as described previously.[15] The caveolin-1 and the phospho-caveolin-1-specific antibodies (clone 2297 and P-Tyr14, Transduction Laboratories) were diluted 1:1000 and 1:2500 in TBST, respectively. To ensure equal loading, blots were stripped in 200 mmol/L glycine, 1% Tween-20, 0.1% sodium dodecyl sulfate for 2 hours at room temperature, incubated with an actin-specific monoclonal antibody (clone C4, Roche) diluted at 1:5000 and developed.

Results

A Microarray-Based Analysis Identifies Caveolin-1 (CAV1) as Differentially Expressed in Ovarian Carcinoma

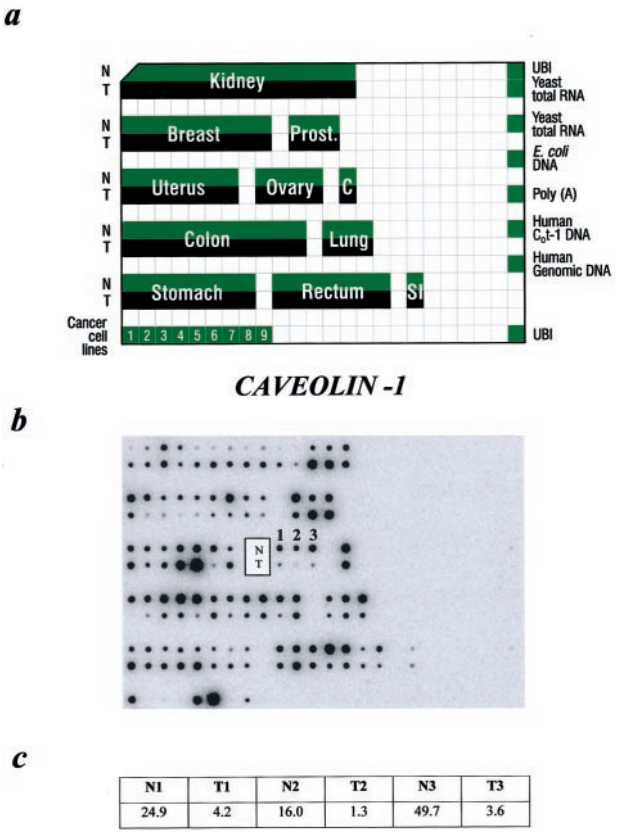
To search for novel markers differentially expressed in human ovarian cancer as compared to normal tissue, we used the Atlas Select human tumor cDNA array representing 437 genes selected as being up- or down-regulated in various human cancer tissues (Clontech; *www.clontech.com/archive/oct99upd/atlasselect.html*). We hybridized the arrays with total RNA obtained from two normal human ovaries and two grade 3 serous ovarian carcinomas. This analysis revealed 16 genes overexpressed and 41 genes down-regulated by a factor of 2 or more in both tumor samples (data not shown). Among the down-regulated genes, we observed a strong suppression of the *CAV1* gene with relative expression levels in the carcinoma samples as compared to the normal samples of 0 and 0.09, respectively.

A *CAV1*-specific probe was then hybridized onto an array containing 68 cDNA pair samples derived from multiple human tumors and corresponding normal tissue from individual patients[19] (matched tumor/normal array; *http://www.clontech.com/archive/jan00upd/pdf/matchedarray.pdf*). As shown in Figure 1, *CAV1* is strongly suppressed in three of three tumors derived from ovary, but also in breast (nine of nine) and colon (eight of eleven). In contrast, a significant up-regulation of *CAV1* is observed in eleven of fifteen tumor samples derived from kidney, in prostate (two of three), and in stomach (six of eight) when compared to the corresponding normal cDNA.

Caveolin-1 Is Down-Regulated at the Protein Level in Cell Lines and Ovarian Carcinomas

Using Western blot analysis, high levels of caveolin-1 were detected in immortalized human ovarian surface epithelial cells, in SKOV-3 and ES-2 ovarian cancer cell lines and in protein extracts prepared from two benign serous adenomas (Figure 2a). However, no caveolin-1 expression was present in the MDAH 2774, CaOV-3, OVCAR-3, and in OAW42 carcinoma cells.

Subsequent immunohistochemical analysis (summarized in Table 1) revealed a strong, fine-granular expression pattern of caveolin-1 concentrated at the basal and lateral membranes of the normal ovarian surface epithelium. In addition, caveolin-1 expression was present in the cortical stroma underlying the surface epithelium (Figure 2c). In benign serous cysts and adenomas a similar basolateral membrane-associated expression was detectable (Figure 2d), whereas in the epithelium of



N1	T1	N2	T2	N3	T3
24.9	4.2	16.0	1.3	49.7	3.6

Figure 1. Expression analysis of *CAV1* using the matched tumor/normal array. **a:** Organization of the cDNA samples derived from tumor and normal tissue of individual patients on the array. N, normal; T, tumor. Numbers 1 to 9 identify cDNAs derived from the following cell lines: 1, HeLa; 2, Daudi; 3, K562; 4, HL60; 5, G361; 6, A594; 7, Molt4; 8, SW480; 9, Raji. **b:** Hybridization results obtained with the *CAV1* probe. The filters used contain three ovary samples (serous type adenocarcinomas) indicated in the figure by N (normal) and T (tumor) and numbers 1 to 3. **c:** Relative *CAV1* expression levels as obtained by phosphoimage analysis in the three ovary-derived cDNA pairs are shown in the table.

seven mucinous adenomas analyzed, caveolin-1 was completely absent (data not shown). Grade 1 carcinomas and serous tumors of borderline malignancy, expressed reduced levels of caveolin-1. In these tumors, the membrane-bound caveolin-1 expression present in the normal epithelium was altered and the protein seemed evenly distributed through the whole cytoplasm (Figure 2e). Most interestingly, caveolin-1 staining was significantly reduced in 29 grade 2 and 3 serous and endometrioid ovarian carcinomas (Figure 2f; Table 1).

Caveolin-1 Is Phosphorylated in SKOV-3 and ES-2 Carcinoma Cells

Caveolin-1 was detected as a tyrosine-phosphorylated protein in v-src-transformed cells[20] and recently Lee and colleagues[21] showed that caveolin-1 undergoes phosphorylation at tyrosine 14 in response to growth factor signaling. To assess whether such a modification also occurs in ovarian carcinoma cells, we performed Western blot analysis with extracts derived from normal ovarian epithelial cells and from SKOV-3 and ES-2 carcinoma cells using a phospho-caveolin-1-specific antibody. As shown in Figure 2b, the 22-kd isoform of caveolin-1 is phosphorylated at tyrosine 14 in both carcinoma cell lines, however, not in the immortalized epithelial cells.

Down-Regulation of Caveolin-1 Involves Hypermethylation

The *CAV1* gene is located on chromosome 7q31.1.[22] Loss of heterozygosity has been described within this region however, currently there is no evidence for deletions of the *CAV1* gene in human tumors.[17] To investigate the mechanism responsible for down-regulation of *CAV1* in ovarian carcinoma cells, we used inhibitors of DNA methylation (5-aza-2′deoxycytidine) and histone deacetylation (TSA). In addition, we also analyzed the influence of RAS-dependent signaling pathways on *CAV1* expression by interfering with MEK1/2 (MAP/ERK kinase) and PI-3 kinase (phosphatidylinositol-3 kinase) using the PD98059 and LY294002 inhibitors, respectively.

A strong up-regulation of caveolin-1 was observed both after 5-aza-2′deoxycytidine and TSA treatment in OVCAR-3 and OAW42 cells (Figure 3, a and b). Inhibition of DNA methylation by 5-aza-2′deoxycytidine resulted in re-expression of both CAV1 isoforms, whereas inhibition of histone deacetylation by TSA had a weaker effect that seemed to be isoform-dependent. In OAW42 cells, a slight up-regulation of the smaller 22-kd caveolin-1 protein was also detectable after inhibition of MEK1/2 by PD98059. This inhibitor did not alter caveolin-1 expression in OVCAR-3 cells and no effect of PI-3 kinase repression by LY294002 was observed.

To distinguish between effects directly controlling expression of the *CAV1* gene and indirect effects resulting in caveolin-1 protein expression, we performed Northern blot analysis from the same cell lines after treatment with 5-aza-2′deoxycytidine and TSA. As shown in Figure 3c, inhibition of DNA methylation resulted in up-regulation of the *CAV1* mRNA in OVCAR-3 and OAW42 cells (Figure 3c, compare lanes 1 to 3 and lane 5 to 7, respectively). Only in OAW42 cells, a stimulation of mRNA expression after inhibition of histone deacetylation by TSA was observed, but not in OVCAR-3 cells (Figure 3c, compare lanes 1 to 4 and lane 5 to 8, respectively). In both cell lines, the differences in expression levels observed at the protein level were not fully reflected at the mRNA level.

Forced Expression of CAV1 Reduces Survival of OVCAR-3 Ovarian Carcinoma Cells

To examine a functional involvement of *CAV1* in growth regulation of ovarian cancer cells, we introduced the gene into OVCAR-3 cells. In a colony formation assay, growth of G418-resistant clones harboring the *CAV1* (pLNHX-cav) expression construct or the control vector (pLNHX) was measured. *CAV1* expression resulted in a reduction of colony formation of OVCAR-3 cells of ~70% (Figure 4A). Analysis of G418-resistant clones obtained after transfection with the pLNHX-cav plasmid revealed that none of these clones expressed detectable levels of

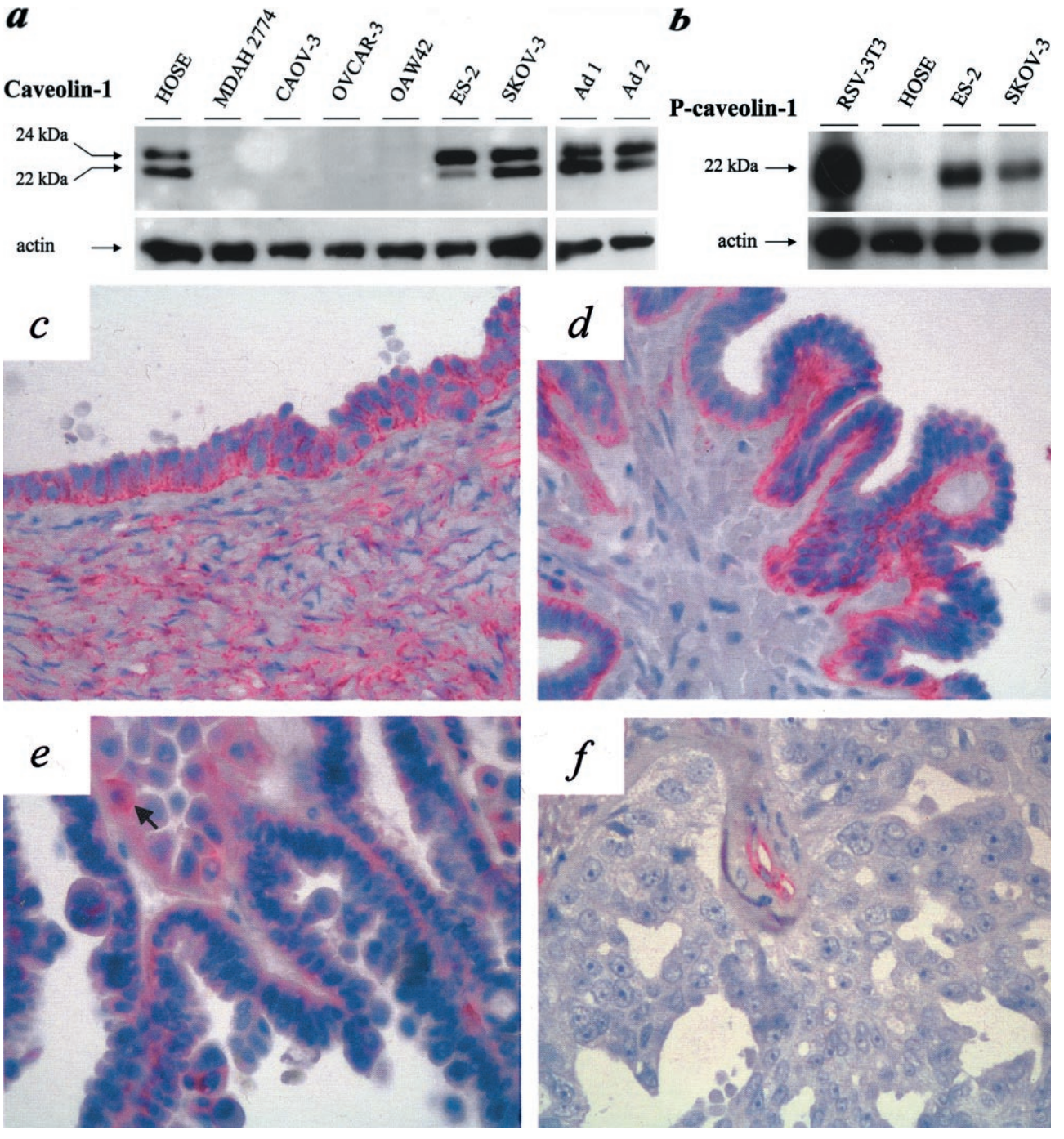


Figure 2. Western blot and immunohistochemical analysis of caveolin-1 expression in human ovarian carcinoma cells. **a:** In human ovarian surface epithelial cells (HOSE), in ES-2 and SKOV-3 human ovarian carcinoma cells the α (22 kd) and β-isoforms (24 kd) of caveolin-1 are present, no expression is detected in MDAH2774, CAOV-3, OCVAR-3, and OAW42. Both isoforms are also present in protein preparation of two serous ovarian adenomas (Ad1, Ad2). **b:** The use of a phospho-caveolin-1-specific antibody shows Tyr-14 phosphorylation of the caveolin-1 α-isoform in tumor cell lines ES-2 and SKOV-3. A lysate from RSV-3T3 mouse fibroblasts expressing pp60src was used as a positive control. Blots were stripped and reprobed with an antibody against cytoplasmic actin to control for equal loading. **c:** Immunohistochemical analysis of caveolin-1 shows expression in the surface epithelium and in the underlying stroma of normal ovary. **d:** A similar staining is evident in the epithelial lining of a serous adenoma, whereas loss of the membrane-associated localization and down-regulation can be observed in a grade 1 serous carcinoma as depicted by the **arrow** (**e**). **f:** Complete loss of caveolin-1 expression from the epithelial cells is observed in a grade 3 serous carcinoma. Positive staining of blood vessel endothelial cells serves as an internal positive control.

caveolin-1 protein (data not shown). Therefore, we used a different CAV-1 construct (pc-CAV-V5) in which a cytomegalovirus promoter induces high levels of caveolin-1 expression after transient transfection (Figure 4B). Selection of stable clones harboring the pc-CAV-V5 construct revealed >90% suppression of colony formation indicating that high levels of caveolin-1 expression are incompatible with survival in OVCAR-3 cells (Figure 4C). In these experiments we had noticed increased amounts of detached cells 48 hours after transfection. When we per-

Table 1. Expression of Caveolin-1 Protein in Human Ovarian Tissues

Ovarian specimen	Histology	Number of samples	Mean staining intensity	% of stained cells	Mean score
Normal ovary		6	3.0	93	278
Benign	Serous cysts/adenomas	19	2.9	83	247
	Mucinous adenomas	7	0.0	0	0
Borderline	Serous	5	2.0	17	36
Carcinoma (grade 1)	Serous	3	1.3	10	15
	Mucinous	2	0.0	0	0
Carcinoma (grade 2)	Serous	10	0.3	3	5
	Endometrioid	3	0.7	6.7	7
Carcinoma (grade 3)	Serous	16	0.4	5.3	6

formed a TUNEL assay in OVCAR-3 cells transiently transfected with either the pc-CAV-V5 construct or the empty vector pc-V5, the number of apoptotic cells was significantly increased in the CAV-1-transfected cells as compared to the control transfectants (Figure 4D).

Thus, the results obtained by array hybridization, immunohistochemistry, and transfection studies suggest an important role of caveolin-1 in the survival of human ovarian epithelial cells.

Discussion

The *CAV1* gene, which encodes a major component of membrane caveolae and an important signaling regulator, was consistently down-regulated at the level of RNA and protein in ovarian carcinomas. In serous ovarian tumors, we observed a gradual loss of protein expression with increasing de-differentiation. Whereas normal ovaries and most serous adenomas expressed comparable levels of caveolin-1, all mucinous adenomas analyzed were completely negative for caveolin-1 expression. These two types of ovarian tumors most likely develop by different molecular mechanisms as indicated by the observations that *KRAS* mutations are more frequently detected in mucinous ovarian carcinomas.[23,24] More importantly, Ono and colleagues[25] identified more than 100 genes differentially expressed between serous and mucinous type ovarian carcinomas. This suggests that the *CAV1* gene belongs to a larger group of genes involved in the control of signaling and growth preferentially in serous-type ovarian epithelial cells.

In addition to *CAV1* down-regulation in ovarian carcinomas, we observed a decreased expression of *CAV1* in tumors derived from breast, colon, lung, and stomach using the matched tumor/normal array. These observations confirm data published by others,[14,26] however care

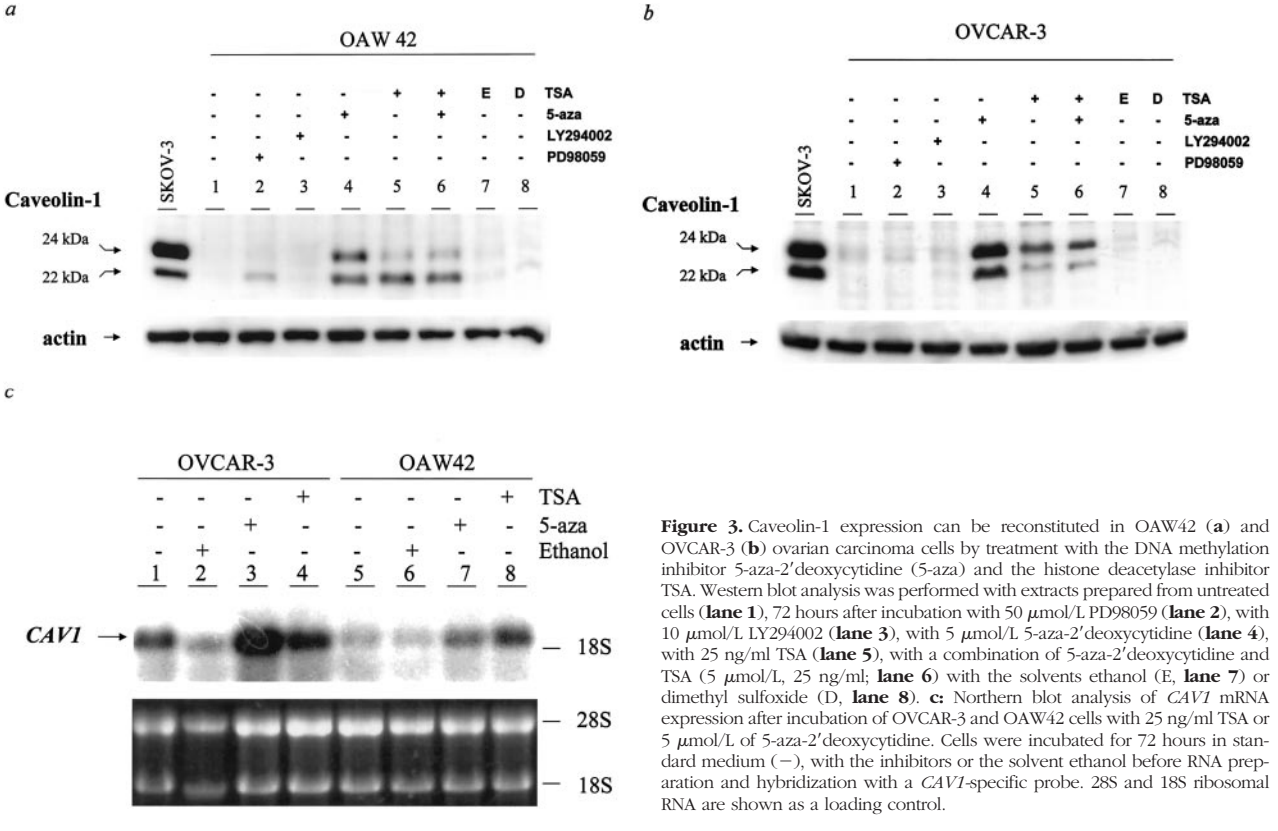


Figure 3. Caveolin-1 expression can be reconstituted in OAW42 (**a**) and OVCAR-3 (**b**) ovarian carcinoma cells by treatment with the DNA methylation inhibitor 5-aza-2′deoxycytidine (5-aza) and the histone deacetylase inhibitor TSA. Western blot analysis was performed with extracts prepared from untreated cells (**lane 1**), 72 hours after incubation with 50 μmol/L PD98059 (**lane 2**), with 10 μmol/L LY294002 (**lane 3**), with 5 μmol/L 5-aza-2′deoxycytidine (**lane 4**), with 25 ng/ml TSA (**lane 5**), with a combination of 5-aza-2′deoxycytidine and TSA (5 μmol/L, 25 ng/ml; **lane 6**) with the solvents ethanol (E, **lane 7**) or dimethyl sulfoxide (D, **lane 8**). **c:** Northern blot analysis of *CAV1* mRNA expression after incubation of OVCAR-3 and OAW42 cells with 25 ng/ml TSA or 5 μmol/L of 5-aza-2′deoxycytidine. Cells were incubated for 72 hours in standard medium (−), with the inhibitors or the solvent ethanol before RNA preparation and hybridization with a *CAV1*-specific probe. 28S and 18S ribosomal RNA are shown as a loading control.

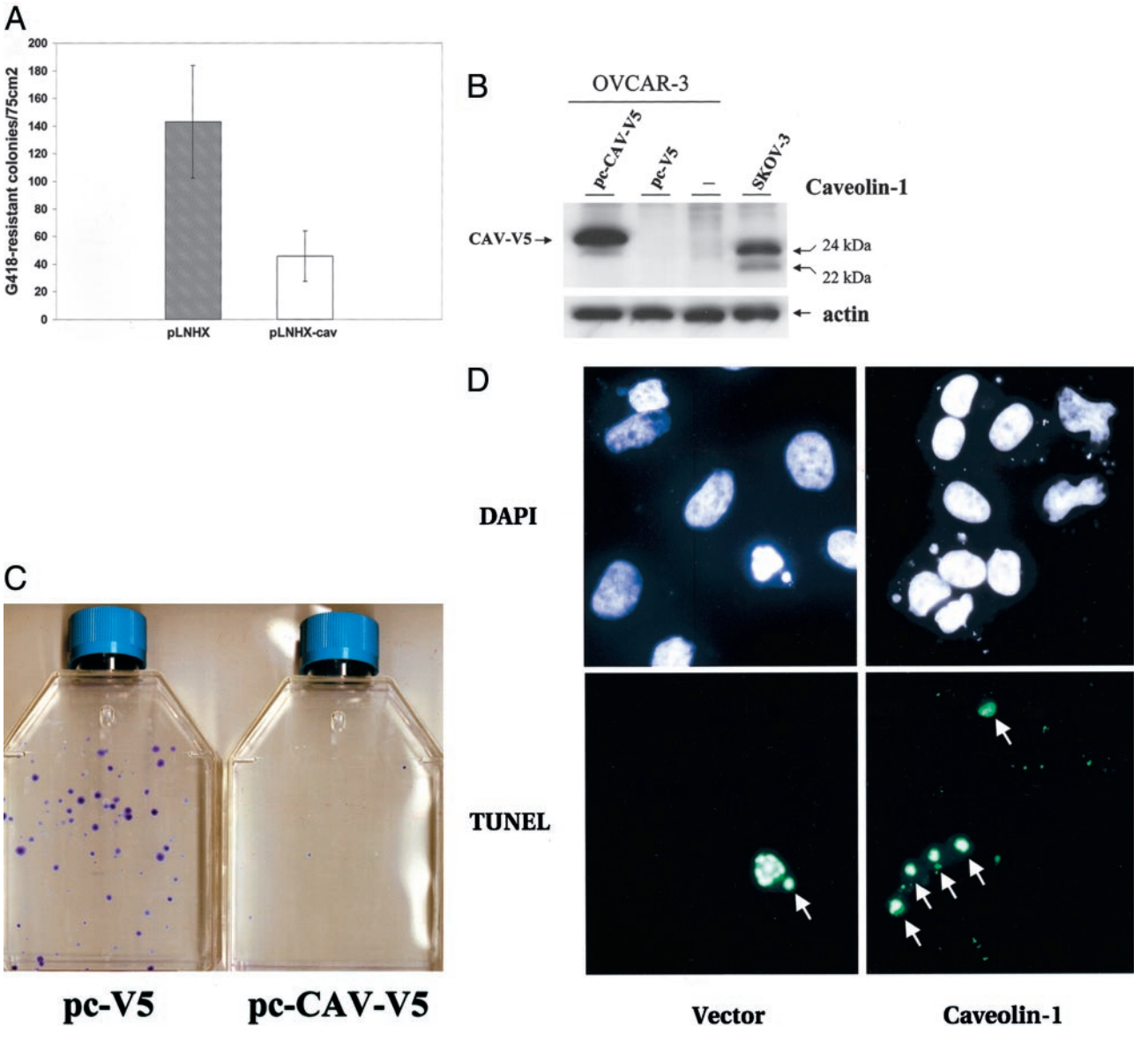


Figure 4. A: Colony formation assay demonstrating a strong reduction of survival in OVCAR-3 cells by expression of *caveolin-1*. OVCAR-3 cells were transfected with either the *caveolin-1*-expressing vector (pLNHX-cav) or the empty vector (pLNHX). Cells were selected in 0.5 mg/ml of G418 until colonies were visible. After fixation and staining, colonies were counted and values are displayed as colonies/flask (75 cm^2). The mean range and SD of four independent experiments is shown. **B:** High expression of caveolin-1 protein in OVCAR-3 cells after transient transfection of pc-CAV-V5. Cells were either transfected with pc-CAV-V5, the empty plasmid pc-V5 or left untransfected (−) before preparation of protein extracts. The V5-epitope-tagged caveolin-1 expression construct encodes a protein of a ~32 kd. SKOV-3 cells were used as a positive control and actin was used as a loading control. **C:** Colony formation assay using the pc-CAV-V5 construct or the empty plasmid pc-V5 in OVCAR-3 cells. Zeocin-resistant colonies were selected for 2 weeks, fixed, and stained. **D:** Overexpression of pc-CAV-V5 increases apoptosis in OVCAR-3 cells. OVCAR-3 cells were transiently transfected with pc-CAV-V5 (caveolin-1) or the empty pc-V5 (vector). DNA fragmentation assay (TUNEL) was performed 48 hours later and nuclei were stained using diamidinophenylindole (DAPI). **Arrows** show TUNEL-stained cells, indicative of apoptosis.

has to be taken in the interpretation of array-derived data because of the high expression of caveolin-1 mRNA and protein in normal mesenchymal and endothelial cells detected *in situ*.[15] Using immunohistochemistry we confirmed loss of caveolin-1 expression from the ovarian epithelium on tumor progression. In other tissues such as breast and lung, expression of the caveolin-1 protein in the epithelial cells *in situ* has not yet been demonstrated. In normal resting breast tissue caveolin-1 expression rather seems to be restricted to the myoepithelial cells, whereas the secretory epithelial cells are negative[17] (Wiechen and Sers, unpublished observations). In addition, the mRNA expression might not always reflect protein expression within a distinct cell type. Therefore, a combination of array hybridization, immunohistochemical, and functional analysis is crucial to define a role for caveolin-1 in the growth regulation of distinct human tumors.

CAV1 was found up-regulated in tumor-derived cDNAs prepared from kidney, and prostate, suggesting an entirely different function of the gene in these tissues. Involvement of caveolin-1 in prostate carcinoma progression has been described by Yang and colleagues,[16] who

suggested that the caveolin-1 protein plays a role in prostate cancer progression and protects prostate cancer cells from Myc-induced apoptosis.[16,27] Thus, depending on the cellular context, caveolin-1 seems to have opposing functions that result either in growth inhibition or growth promotion. Such alternative activities are also known for other gene products, eg, the RAS protein that, by using similar signaling pathways, can induce either differentiation or transformation in distinct cell types.[28] Whereas the majority of ovarian carcinoma cell lines and tumors display down-regulation of caveolin-1, SKOV-3 and ES-2 cells express high levels of caveolin-1 despite their unlimited growth capacity. One explanation for this seemingly contradictory finding is the phosphorylation of the α-isoform of caveolin-1 on tyrosine 14 in these cells. Growth factor-dependent phosphorylation of caveolin-1 stimulates its capacity to bind the Grb7 adapter protein and thereby enhances anchorage-independent growth in a cell type-specific manner.[21] The observation that SKOV-3 and ES-2 ovarian carcinoma cell lines display high levels of phosphorylated caveolin-1 allows two potential interpretations: either the cells have developed mechanisms to circumvent caveolin-1-mediated growth suppression, or the specific phosphorylation of caveolin-1 as a consequence of growth factor receptor overexpression results in an augmentation of signaling in these cells.

In contrast, in OVCAR-3 cells that have the *CAV1* gene suppressed, caveolin-1 overexpression resulted in increased apoptosis. Direct induction of programmed cell death by caveolin-1 has not been described and whether this is a cell type-specific effect remains to be investigated. A potential involvement of *CAV1* in the regulation of apoptosis was demonstrated recently in fibroblasts and T24 human bladder carcinoma cells. Anti-sense-mediated suppression of *CAV1* abrogated the sensitivity of these cells toward apoptotic stimuli.[29,30] Inhibition of the PI-3 kinase pathway by LY294002, restored this sensitivity and overexpression of caveolin-1 repressed the activity of PI-3 kinase. OVCAR-3 cells harbor increased levels of PI-3 kinase activity[31] and the simultaneous expression of high levels of caveolin-1 could initiate conflicting signals that result in cell death. Activation of the PI-3 kinase pathway and amplification of *AKT2* have been shown in a significant proportion of ovarian carcinomas.[31,32] The potential role of caveolin-1 in apoptosis sensitization or even induction by interfering with PI-3 kinase activity points toward a specific role of caveolin-1 in the suppression of ovarian carcinoma development.

The gene encoding caveolin-1 is located on human chromosome 7q31.1.[22] Loss of heterozygosity on 7q31.1 has been detected in carcinomas of ovary,[33] prostate,[34] stomach,[35] and kidney,[36] suggesting loss of *CAV1* in human ovarian cancer. However, deletions or mutations within the *CAV1* gene were not detected.[17] Methylation of CpG islands in the *CAV1* promoter region was observed only in human breast cancer cell lines[37] but not in ovarian cancer samples.[17] This suggests, that CAV1 might act as a class II tumor suppressor gene. Class II tumor suppressor genes are characterized by a block in their expression rather than by mutations or deletions.[38]

Our data obtained after treatment of the ovarian carcinoma cell lines with 5-aza-2′deoxycytidine suggested that methylation of regulatory regions of the *CAV1* gene is one mechanism to suppress *CAV1*. Persistent tumor suppressor gene silencing is accomplished by the concerted action of CpG methylation and histone deacetylation.[39] Therefore the observation that caveolin-1 protein expression can be effectively induced using either 5-aza-2′deoxycytidine or the histone deacetylase inhibitor TSA was expected. However, investigation of the *CAV1* mRNA expression in response to 5-aza-2′deoxycytidine and TSA revealed that mainly de-methylation of the DNA-stimulated expression of the *CAV1* gene in OVCAR-3 and OAW42 cells. In OAW42 cells, inhibition of histone deacetylation could also activate *CAV1* mRNA expression to some extent, not in OVCAR-3 cells. A complex regulation of the *CAV1* gene was also described by Li and colleagues[40] who investigated the mechanisms mediating up-regulation of caveolin-1 in prostate carcinoma cells by testosterone. Whereas the caveolin-1 protein was significantly up-regulated by testosterone, the response of the *CAV1* promoter to this stimulation was rather low. These data and our own observations suggest, that in addition to promoter methylation and histone deacetylation, expression of the caveolin-1 gene and protein might be further regulated at a posttranscriptional level.

In summary, by using a combination of two different cDNA arrays hybridized with material prepared from human tissues, immunohistochemical analysis, and functional investigation we have identified *CAV1* as a strong candidate tumor suppressor in human ovarian carcinoma.

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